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Screening and management of type 2 diabetes mellitus with Raman spectroscopy

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Raman spectroscopy is a well-established analytical chemistry technique, which has proven to have numerous medical applications because of its chemical fingerprinting capabilities and compatibility with biological samples. Surface-enhanced Raman spectroscopy (SERS), an embodiment of Raman, has overcome many of the major limitations of normal Raman and has expanded its medical avenues due to its low detection limits. A growing application of Raman and SERS in clinical research focuses on the screening and management of type 2 diabetes mellitus (T2DM) to improve patient outcomes and quality of life. This review examines the Raman and SERS effects and outlines a few of their major applications (e.g., diagnosis, glucose measurement, biomarker detection, and pathogen identification) and current challenges (e.g., calibration for anatomical differences and inconsistencies of glucose levels in blood and interstitial fluids) within T2DM clinical studies.

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1 Raman Spectroscopy

1.1 The Raman effect

The Raman effect was named after the Indian physicist Sir Chandrasekhara Venkata Raman (C V Raman), who reported the first qualitative Raman findings on March 31, 1928 [1]. C V Raman observed several natural phenomena, such as the blue color of the Mediterranean Sea and the scattering of blue light by Alpine glaciers, which he explained by variances in photon scattering [1]. For this work in light scattering and the discovery of the Raman effect, C V Raman was awarded the 1930 Nobel Prize in physics [2]. It belongs to one of the four most important discoveries in physics with the highest impact to applications in modern life [3]. After the discovery of Raman Effect, researchers all over the world became interested in this new technique, which is based on the inelastic scattering of light. In his Nobel laureate lecture, he stated that the Raman effect "opened up an illimitable field of experimental research in the study of the structure of matter" [2]. This has proven true, especially in the medical field, where non-destructive analysis of complex biological samples is now attainable. In the Raman effect, one (1) out of the million (10^6) photons incident on a molecule spontaneously exhibits inelastic scattering, where the incident and scattered photons differ in energy (Fig 1). This energy difference offers a chemical fingerprint of the analyte's structure through its translations into characteristic molecular vibrations [4].

The two types of Raman scattering are Stokes (the scattered light with lower energy as compared to the incident light) and anti-Stokes (the scattered light with higher energy compared to the incident light) (Fig 1), which are associated with excited electrons returning to a vibrational state or ground state, respectively.

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Stokes Raman scattering has the highest incidence, where the electrons are excited from the lowest ground state and relax back to a vibrational state (ν_i) above the lowest ground state (S_0) [5]. The Stokes Raman scattering occurs more often because most electrons in the analyte will already be in the ground state. The use of a monochromatic laser radiation (e.g., 532 nm, 632 nm, and 785 nm) to bombard the analyte with photons stimulates the spontaneous Raman scattering. The intensity of anti-Stokes bands mirroring the position of Stokes bands is lower because fewer electrons are initially present in electronic states above the ground state [6]. To target anti-Stokes Raman shifts, the temperature of the analyte can be raised to excite electrons to a vibrational state. The intensity of both the Stokes scattering and anti-Stokes Raman scattering can be further enhanced by multiple magnitudes (up to 10^6) through the resonance Raman effect, where the virtual state is actually an electronic excited state (S_1) [7].

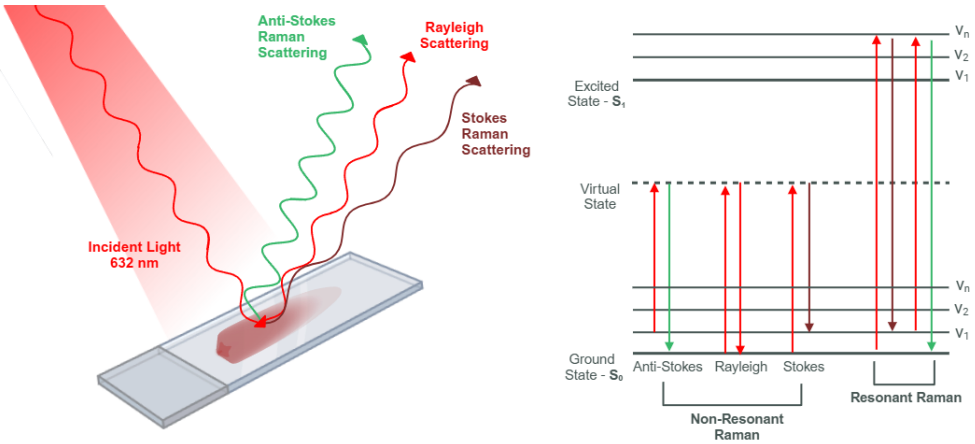

Fig 1. A schematic of different types of Raman scattering (left) and the related Jablonski diagram (right) [8]. This figure was created using BioRender.

1.2 Raman active molecules and their spectra

Raman spectroscopy measurements can be carried out in any phase of matter. In modern dispersive spectrometers, Raman shifts are separated with the help of a diffraction grating and typically recorded with a silicon CCD detector that produces the Raman bands of various intensities (i.e., the Raman spectrum).

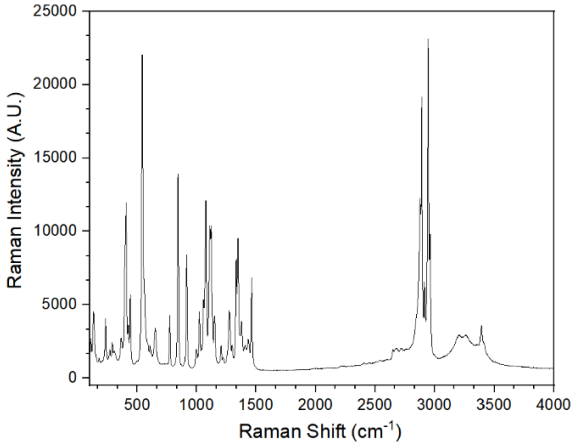

Fig 2. A micro-Raman spectrum of solid D-glucose anhydrous recorded using a LabRam HR800 system, after excitation with a HeNe laser at 632.8 nm.

The presence, absence, or change in Raman spectral features such as the Raman shift or wavenumber (cm^{-1}) characteristic to molecular vibrations can be used to elucidate the chemical fingerprint of analyte molecules. Raman-active analytes must exhibit polarizability changes during the movements of their chemical bonds (e.g., stretching and bending), where the electron cloud undergoes a net disturbance during the excitation with a laser light. The intensity of Raman scattering is proportional to the degree of polarizability, where the chemical bonds with strong dipoles (e.g., C=O and O-H) have weak Raman signals and bonds with weak dipoles are strongly polarizable (e.g., C=C and C-H) [8]. The fingerprint region of a Raman spectrum is where the key structural and biological shifts are typically located (Fig 2 – below 2000 cm^{-1}). Raman shifts above 2000 cm^{-1} are commonly associated with vibrational modes characteristic to solvents such as water and ethanol, and stretching modes of N-H, S-H, and C-H groups in biomolecules [9,10].

1.3 Advantages and disadvantages of Raman spectroscopy

Raman spectroscopy is one of the fastest growing analytical chemistry techniques due to its unique advantages. In Raman, molecules of interest can be "vibrationally fingerprinted" with minimal to no sample preparation, in any physical state or aqueous solutions, and noninvasively (Fig 3). However, the standard lab bench Raman setup requires a dedicated imaging space to maintain an ideal temperature, low humidity level, and in most instances absence of light during data collection. These prerequisites are a challenge for expansion into fields that did not possess the resources for dedicated space, specialized equipment, and trained operators for complex data analyses. While Raman spectrometers were initially in benchtop form, the production of portable and handheld spectrometers has made the technique more accessible for field testing and other analysis outside of laboratory settings. With the option of using near infrared or infrared laser wavelengths, highly sensitive and non-destructive Raman analyses of samples limited in quantity or susceptible to photodamage (e.g., cells, historical samples, and forensic samples) also become possible.

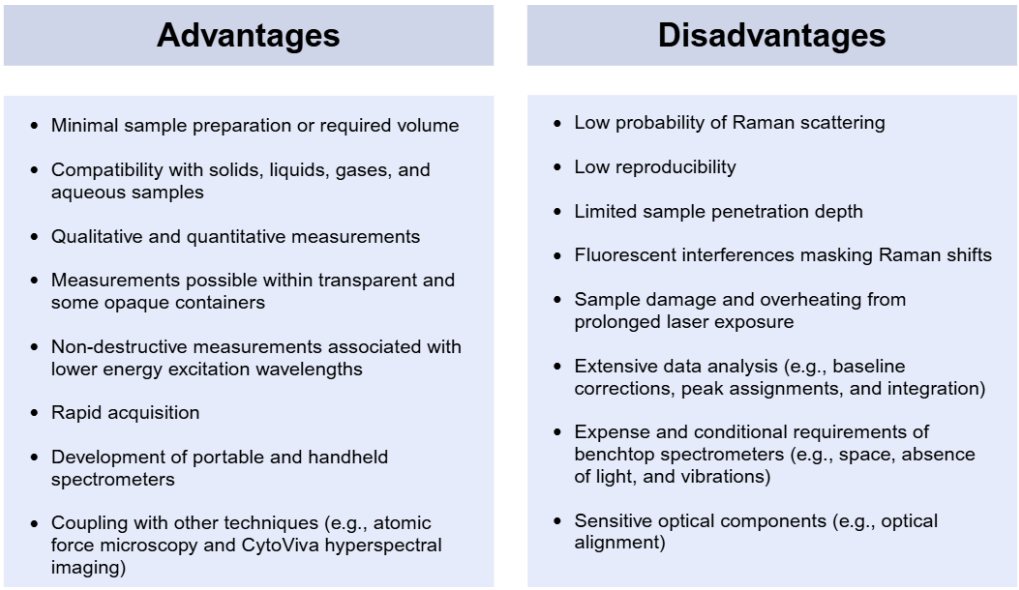

Fig 3. Schematic of the advantages and disadvantages of normal Raman spectroscopy [11].

Some of the major Raman disadvantages (e.g., low sensitivity, fluorescence interferences, and sample photodamage) can be overcome by an embodiment of Raman spectroscopy, namely surface enhanced Raman spectroscopy (SERS). The SERS effect was discovered over 50 years ago and has found numerous biomedical applications since then. In the medical field, SERS has been extensively exploited due to its

biosensing and bioimaging capabilities at low biomolecule concentrations (e.g., DNA, RNA, microRNA, cancer biomarkers, pathological cellular markers, and neurotransmitters) [12-14]. In SERS, a nanometallic substrate (e.g., gold and silver nanoparticles) can enhance the intensity of Raman scattering by up to 10^{11} orders of magnitude, down to the single molecule level of detection [15-17]. SERS requires the presence of analytes nearby or at the nanoscale interstitial sites of metallic nanostructures. These favorable spatial locations for exciting intense SERS are often referred to as SERS "*hot spots*" and largely arise due to the increase in the magnitude of both the incident and the scattered electromagnetic fields associated with the excitation of coupled, localized surface plasmon resonances (LSPR) of the nanostructures (Fig 4). This is known as the electromagnetic enhancement mechanism or physisorption and is probably the largest contributor to the overall SERS enhancement [18]. Physisorption offers electromagnetic enhancements due to the very close vicinity of analytes to the nanosubstrate (< 5 nm) without interacting chemically [19]. On the other hand, the chemisorption mechanism offers charge transfer enhancements through the direct chemical complexation of biomolecules to the nanosurface (i.e., new bonds such as Ag-O or Ag-S) [19]. Other factors influencing the SERS enhancement are the size, shape, and homogeneity of the nanostructures, the laser excitation wavelength, and the dielectric environment.

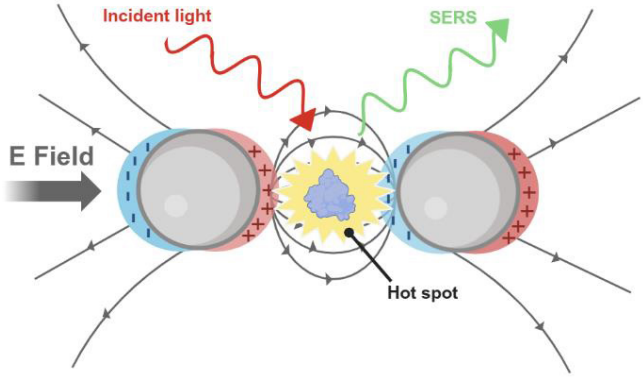

Fig 4. A depiction of an analyte molecule within a SERS hotspot of two silver nanoparticles [18]. This figure was created using BioRender.

1.4 Raman spectroscopy in clinical settings

Nowadays, clinics and hospitals use Raman spectrometers as an on-site, non-invasive, diagnostic tool. Current topics receiving attention in clinical Raman-based research studies are early detection of cancer, defining surgical margins, disease biomarker detection, and identification of pathogens [10,20,21]. This expansion is owed to the Raman's compatibility with biological samples, rapid acquisition, non-destructive nature, and automation features with molecular discrimination capabilities in newer spectrometers. To circumvent delays from sending collected biopsies or cultures to an external facility, Raman can be used in-house to expedite patient care. Raman spectroscopy is an underexplored tool in the routine monitoring and prognosis of patients with chronic diseases such as type 2 diabetes mellitus. As the global population ages, the number of individuals living with chronic illness is growing, warranting for the fast, non-invasive analysis that Raman spectroscopy offers [22].

2 Type 2 Diabetes Mellitus (T2DM)

As of 2021, type 2 diabetes mellitus (T2DM) was the eighth leading cause of death in the United States (U.S.), with an estimated 830 million individuals living with the disease globally [23]. With 1 in 3 Americans (97.6 million) being diagnosed with prediabetes and 11.6% of the US population with diabetes (38.4 million), the direct cost for medical care has risen to $237 billion in 2022 [24]. T2DM has the highest

incidence (90-95%), but there are multiple variations of diabetes mellitus including type 1, type 2, gestational, and diabetes attributed with secondary causes [25,26]. While type 1 diabetes is understood as an autoimmune reaction targeting the pancreas, T2DM is a chronic metabolic disease driven by reduced insulin secretion from pancreatic β-islet cells with systemic insulin resistance (Fig 5) [27]. Insulin activates the process of glucose uptake for storage as glycogen, so the absence (T1DM) or resistance (T2DM) to insulin consequentially leads to chronic hyperglycemia. Lifestyle is a known determinant of T2DM (e.g., low physical activity, highly processed diet, and obesity), but other risk factors include genetics, family history, hormonal abnormalities, damage to the pancreas, and certain medications [28]. The pathophysiology of T2DM is multifactorial, and has complications of chronic inflammation, dyslipidemia, peripheral neuropathy, peripheral vascular disease, lower limb amputation, skin infection, renal failure, and blindness [29,30]. T2DM is also closely associated with cardiovascular diseases (e.g., atherosclerosis, coronary artery disease, hypertension, myocardial infarction, and stroke) and has been recently linked to Alzheimer's disease [23,29,31].

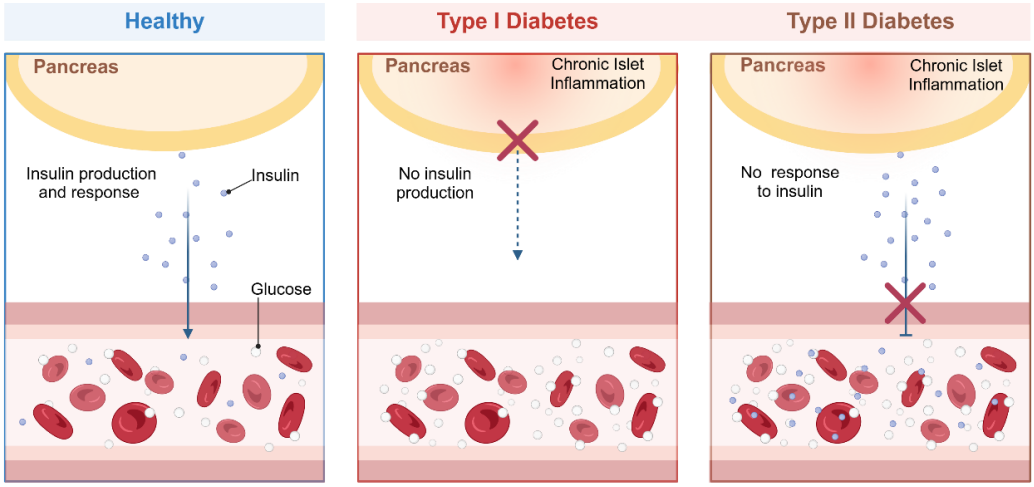

Fig 5. Mechanisms leading to type 1 (left) and T2DM (right). This figure was created in BioRender.

Patients living with T2DM face one of the most extensive routines to maintain and improve their condition. This includes daily glucose monitoring, adherence to medications to decrease blood glucose levels (e.g., metformin), dietary adjustments to avoid glucose spikes, physical activity, checking peripheral limb circulation, having a periodic A1C test, taking great caution to avoid trauma or injury to lower the chances of infection, and wound care to manage current injuries [32]. It is also commonplace and recommended for patients with T2DM to have additional screenings to gauge cholesterol, organ damage (e.g., kidneys, liver, and pancreas), thyroid stimulating hormone levels, lower extremity exams, and eye exams [32]. Though not in vain, these tests can prove overwhelming, painful, and increase the risk of infection for patients. The high sensitivity of Raman spectroscopy, down to differences in a single amino acid residue, offers a viable alternative and supplement to current strategies to improve the quality of life in patients living with T2DM [33,34].

3 Glucose monitoring and diagnostic screening

The most widespread application of Raman spectroscopy in T2DM studies are diagnostic screening and glucose monitoring. Diagnosis of diabetes is done primarily through the fasting plasma glucose test, random plasma glucose test, the glucose tolerance test, and the A1C test [35,36]. However, inaccuracies can occur with certain variations of anemia, other blood disorders, inappropriate timing, fasting requirements,

low sensitivity, and glucometer variations [37]. The high sensitivity of Raman spectroscopy makes screening for T2DM possible. A clinical research study used Raman to screen for T2DM by non-invasively collecting spectra (800-1800 cm^{-1}) behind the left earlobe, the left arm, the left thumbnail, and the left median cubital vein of human patients (15 s exposure with a 785 nm laser) [38]. Because of a lack of large spectral differences between the control and T2DM groups, artificial neural networks and a support vector machine model were used for screening of Raman data with accuracies of 88.9-90.9% and 76.0-82.5%, respectively [38]. Another clinical research study using Raman as a HbA1c probe for diagnosis of T2DM found that the forehead had the most success of diagnosis with 100% sensitivity and 100% specificity at low HbA1c levels, while the fingertip had 100% sensitivity and 80% specificity in detecting high HbA1c levels [39,40].

The current approach patients use to determine glucose levels outside of the clinic is the capillary blood glucose test where a fingertip is punctured with a lancet (≤ 2 mm) to collect blood on a test strip for insertion into a glucometer [41]. Other methods are venous puncture for laboratory processing or continuous glucose monitoring (CGM), where the patient wears a monitor that measures glucose levels within the interstitial fluid [41]. While the capillary glucose test is relatively inexpensive, there is a risk of infection from the lancet injections [42]. Inappropriate use of glucometers may also cause infection from reusing finger sticks, re-puncturing the same site, and taking the test in an environment (e.g., schools, workplaces, and health facilities) where many community-acquired pathogens are found [43]. Patients may also not measure their glucose to avoid the pain or burden of daily testing [42]. A study that surveyed n = 387 patients (269 males and 118 females) about adherence to therapy for T2DM reported that n = 241 (62.3%) of respondents declined regularly checking their glucose levels [44]. Therefore, there is a need for alternative techniques to monitor glucose without posing the risk of infection. Raman spectroscopy has been applied successfully in many studies as an alternative to standard glucose monitoring [42,45]. The first report of measuring physiological glucose by Raman was in 1998, where glucose within the aqueous humor of rabbits was detected [46-48]. This was followed by human aqueous humor from patients with cataracts and the first non-invasive transcutaneous glucose detection by Raman in 2005 [46-49].

As more clinical research applying Raman to quantify glucose emerged, multiple challenges became evident. Key problems encountered by using Raman for glucose quantification are inconsistencies in the blood glucose concentration versus the glucose levels in the interstitial fluid, calibrating for unique skin compositions, bodily fluid turbidity, detection of low glucose concentrations, and Raman-active erythrocyte constituents producing outlier measurements [42,47]. To address these variables, an algorithm that considers the amount and mobility of plasma and interstitial fluid is needed [50]. Another solution is to utilize Raman during capillaroscopy in order to acquire spectra of "micro vessels" near the surface of the skin or human nailfold. This will ensure that major contributions to the Raman spectra are from the blood constituents and there is no need for drawing blood [50-52]. A study reported a R^2 factor of 0.98 and 100% prediction accuracy (n = 12 volunteers) for glucose concentration when acquiring Raman spectra of the nailbed [42]. Unlike the skin, anatomical nail differences were found to not decrease prediction accuracy of glucose concentration [42]. Measuring nailbeds with Raman can also indicate mineral deficiencies that are associated with diabetic retinopathy, hypertension, and thrombosis in patients with T2DM [53].

4 Metabolite profiling and biomarker fluctuations

A newer application of Raman in understanding T2DM are metabolite profiling and biomarker monitoring. While mass spectrometric techniques dominate metabolite profiling, drawbacks of high costs, extensive operator training, vacuum requirements, and destructive measurements have led to the consideration of alternative techniques for metabolite and biomarker detection. Metabolites and biomarkers serve as hallmarks for disease progression, where the status of potential complications and organ damage from T2DM can be predicted. For instance, label-free sensing of pancreatic islet functional damage was

possible through SERS, where insulin levels were reliably detected at 100 pM, with a detection limit of 35 pM. This was achieved through linking insulin to dense pillars of gold nanoparticles [54]. In another research study comparing normal Raman with ELISA, the measurements of insulin concentration showed a strong linear correlation ($R^2 = 0.97$) between the two techniques [54].

Another major complication of T2DM is chronic kidney disease. In 2022, the U.S. Department of Health and Human Services approximated that 1 in 3 adults with diabetes have chronic kidney disease due to vessel damage from glucose excretion [55]. If left untreated, the patient will need dialysis or a kidney transplant. Chronic kidney disease disproportionately affects those living in low and middle-income countries where inexpensive and accurate diagnostic tests are inaccessible [56,57]. Therefore, using Raman as an inexpensive method to assess the risk of chronic kidney disease is valuable. Because Raman can reliably measure multiple bodily fluids, urine was selected in many studies for easier accessibility, larger available volumes than blood, and no risk of infection from invasive sampling techniques [58]. Important molecules excreted in urine to screen for renal health are albumin, creatinine, urea, glucose, and total protein [56]. A clinical research study consisting of n = 20 patients with T2DM and hypertension and n = 20 healthy volunteers found significant spectral differences between urine samples, where the T2DM group had higher protein and glucose peaks. A partial least squares (PLS) regression analysis determined that urea, creatinine, glucose, phosphate, and total protein had high correlation coefficients of $r = 0.98$ [56]. Further PLS-discriminant analysis had a discrimination accuracy of 81.5% [56]. The gold standard biomarker to screen for renal function is albumin, a major indicator for renal complications [59]. This was reported by a 2020 study that measured Raman spectra of artificial urine with five different albumin concentrations [59]. The reported characteristic peaks of albumin were 660 (ν(CS)), 841 (ν(CC), ρ(CH) and ν(NC) in tyrosine), 940 (ν(CCN) and ν(CC)), 994 (phenylalanine), 1317 (δ(CH)), 1430 (δ(CH_2)), and 1634 cm^{-1} (tyrosine) [59-62]. But the highest albumin concentration peak was at 1450 cm^{-1}, which was assigned to a glycine residue within albumin [59]. In these studies, the concentrations for urea and creatinine were also confirmed. For urea, spectral ranges used were 500 to 560 cm^{-1}, 960 to 1043 cm^{-1}, and 1120 to 1192 cm^{-1}, but the only range for creatinine was 650 to 940 cm^{-1} [58]. However, this study noted that the influence of urinary electrolytes (Cl^-, Na^+, and F^-) on the overall Raman spectrum needs to be determined. Another concern was that low pH conditions (pH < 5) and prolonged, frozen storage conditions can influence albumin concentration [59,61,62]. But the urine samples had an average pH of 6.04 and measured the day of collection, so concentration was not influenced [59].

5 Complication status and bacterial identification

Skin complications manifest in 30 to 70% of patients with both type 1 and T2DM [63]. These conditions either present from a direct association with the disease (e.g., acanthosis nigricans and diabetic dermopathy) or indirectly as seen in increased vulnerability to cutaneous bacterial infections. A non-invasive screening technique for this group of T2DM patients would be valuable, and Raman spectroscopy is a promising candidate.

To test this, a clinical study applied Raman spectroscopy for monitoring the phenomenon of collagen glycation in type 2 diabetic mouse models [64]. Though occurring naturally with age, protein glycation is a rapid, nonenzymatic process accelerated by hyperglycemic conditions in T2DM patients, where carbonyl groups of reducing sugars and free amino groups form ketoamine bonds [65-67]. These molecules are reactive and will interact with proteins (e.g., arginine and lysine residues) irreversibly forming advanced glycation end products (AGE), which crosslink and interfere with cell and tissue function [67]. The accumulation of AGEs has a close association with subsequent diabetic complications (e.g., neuropathy, retinopathy, atherosclerosis, nephropathy, and non-alcoholic fatty liver disease), making it an important hallmark of disease progression [68,69]. Accumulation of AGEs in the brain have also been associated with cognitive decline and Alzheimer's disease [70,71]. Because type I collagen is a long-lived matrix protein, it is a frequent target of glycation. In

a recent study, confocal Raman spectroscopy (785 nm excitation laser and 700-1700 cm^{-1} spectral range of acquisition) was applied to collagen scaffolds extracted from T2DM mouse models to report changes in the chemical fingerprint of collagen [66].

The 1005 cm^{-1} peak was attributed to the ring breathing modes of phenylalanine groups, which did not experience significant spectral changes during glycation [66]. The absence of shifts to the amide I (1635 cm^{-1} and 1672 cm^{-1}) and amide III (1244 cm^{-1} and 1274 cm^{-1}) assigned bands indicated the conservation of collagen's triple helix structure, but intensity increased suggesting conformational change to collagen's amino acid background [66]. Other potential markers were proline and hydroxyproline, which compose the second and third amino acid residues of collagen's triple helix, respectively. Peaks assigned to proline (921 cm^{-1}, 1033 cm^{-1}, and 1346 cm^{-1}) increased in intensity, but hydroproline did not undergo significant changes [66]. From these findings, the researchers concluded that confocal Raman spectroscopy is a suitable technique for reporting *in-vivo* AGE accumulation, which previously did not have a standardized method [66]. The surveillance of glycation and AGE deposition on tissues with Raman has the potential for early prediction of complication and disease progression in T2DM patients.

Decreased skin integrity and other pathophysiologic changes from diabetes (e.g., high blood glucose, angiopathy, neuropathy, and immune cell abnormalities) increase the chances of incidence and promote the recurrence of skin infections [63]. The severity of wound infection in T2DM patients is further increased due to delayed healing from chronic inflammation and decreased circulation, lowering immune cell recruitment. In these hyperglycemic environments, the growth of Gram-positive cocci species is favored. This includes *Staphylococcus* (*S.*) *aureus*, a frequent isolate from diabetic wounds and the most common strain responsible for causing osteomyelitis. This complication manifests in 20-60% of diabetic food infections and is the second leading cause of amputation in the U.S. [72-74]. Therefore, it is critical to identify pathogens in a timely manner to administer an effective antibiotic that the strain is susceptible to. Even though data analysis (e.g., interference corrections, calibration, and peak assignment) is a known challenge of Raman spectroscopy, spectral libraries for thousands of biological compounds have been established. A solution is establishing a discriminatory library with the vibrational identities of thousands of relevant bands for immediate identification. This has been accomplished in identifying common pathogens at the single cell level, using SERS. For example, a 2019 research study generated a dataset of n = 30 clinically relevant bacterial strains (94% of pathogens documented in Standford hospitals from 2016 to 2017), and a 2021 study reported n = 22 additional strains [75-78]. By using a convolutional neural network, background noise was eliminated, and identification was achieved with 1 s integration times [75]. The 2019 Raman study reported a strain identification accuracy of 82.2±0.3% using a deep learning approach (convolutional neural network) [75]. Though pathogen identification was successful, physicians must select the correct antibiotic treatment. To address this, the researchers arranged the n = 30 pathogens in groupings by treatment and reported an accuracy of 97.0±0.3% [75]. Furthermore, the high sensitivity of Raman can also discriminate between isolates of *S. aureus* resistant to methicillin and *S. aureus* susceptible to methicillin with 89±0.1% accuracy [75]. With the ability of instant pathogen identification, culturing is no longer needed, delays for patient treatment are reduced, which in turn decreases overall expense and hospital stays [75,79].

6 Perspectives and Conclusions

Since the discovery of the Raman Effect by C V Raman, Raman spectroscopy has experienced major growth with applications in numerous disciplines, including medical science. Raman has been regarded as a promising screening and management tool in the medical field because it can conduct rapid, nondestructive chemical analysis of samples in any state, including aqueous solutions. The low probability of Raman scattering has been overcome by the application of the SERS effect, where sensitivity is enhanced by multiple orders of magnitudes and label-free, single-molecule detection events are possible. Furthermore, instrumental

development leading to compact and portable models have opened doors for its medical applications within hospitals and clinics. A few examples include early cancer diagnosis, pathogen identification, and *in-vivo* Raman-based monitoring during surgical procedures. But a population that could greatly benefit from this non-invasive and sensitive technique are those living with chronic diseases that need routine monitoring. T2DM is one such chronic disease with high occurrence of complication and mortality, yet it has become commonplace globally. The risk for complications largely arises from pathological changes that increase the susceptibility of infection and the risks of lower extremity amputation or even death. To prevent and potentially reduce the insulin resistance responsible for T2DM, patients must adhere to a rigorous treatment plan, that is often burdensome and painful. Raman spectroscopy has proven to be a potential. alternative to many standardized laboratory tests that diagnose T2DM, determine glucose concentration, detect biomarker fluctuations, assess risk for tissue damage and complications, and rapidly identify pathogens. With the help of noninvasive Raman-based analyses, fewer laboratory tests would be required and the chance of acquiring infection from blood draws and glucose monitoring would significantly decrease. Raman and Raman-derived techniques can decrease the time frame from diagnosis to treatment, prevent the spread of an infection, and detect key disease biomarkers that current laboratory tests are not equipped to measure. These current applications and future development of Raman-based techniques have the potential and momentum to change practices of clinical laboratory testing, which may in turn greatly improve the quality of life of patients.

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